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Title

Biosynthesis and *in vitro* enzymatic synthesis of the isoleucine conjugate of 12-oxo-phytodienoic acid from the isoleucine conjugate of α-linolenic acid.

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Abbreviations

AOC, allene oxide cyclase; AOS, allene oxide synthase; COI1, coronatine insensitive 1; 12,13-EOT, allene oxide; GC-MS, gas chromatography-mass spectrometry; 13-HPOT, 13-hydroperoxy octadecatrienoic acid; JA, jasmonic acid; JA-Ile, jasmonoyl-L-isoleucine; JAR1, jasmonic acid-resistant 1; JAZ, jasmonate-zim domain; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LA-Ile, isoleucine conjugate of α-linolenic acid; OPC-8:0, 3-oxo-2-(*cis*-2'-pentenyl)-cyclopentane-1-octanoic acid; OPDA-Ile, isoleucine conjugate of OPDA; OPR, 12-oxo-phytodienoic acid reductase; SCF, skp-cullin-F box.

Abstract

The isoleucine conjugate of 12-oxo-phytodienoic acid (OPDA-Ile), a new member of the jasmonate family, was recently identified in *Arabidopsis thaliana* and might be a signaling molecule in plants. However, the biosynthesis and function of OPDA-Ile remains elusive. This study reports an *in vitro* enzymatic method for synthesizing OPDA-Ile, which is catalyzed by reactions of lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC) using isoleucine conjugates of α-linolenic acid (LA-Ile) as the substrate. *A. thaliana* fed LA-Ile exhibited a marked increase in the OPDA-Ile concentration. LA-Ile was also detected in *A. thaliana.* Furthermore, stable isotope labelled LA-Ile was incorporated into OPDA-Ile. Thus, OPDA-Ile is biosynthesized via the cyclization of LA-Ile in *A. thaliana.*

Keywords

Arabidopsis thaliana, jasmonates, LA-Ile, OPDA-Ile, 12-oxo-phytodienoic acid.

Plants have a wide variety of physiological responses that allow them to adapt to adverse environmental conditions that negatively affect their growth and development. Jasmonic acid (JA, **1**) plays important roles in stress responses and development in plants. JA (**1**) functions as a signaling molecule in numerous plant physiological processes related to development and defense responses.[1] Most enzymes that participate in JA (**1**) biosynthesis have been successfully characterized. JA (**1**) has been shown to be a signaling molecule in both flowering plants and a model lycophyte, *Selaginella moellendorffii*.[2] JA (**1**) is a ubiquitous phytohormone detected in vascular plant species.

The JA (**1**) biosynthetic pathway begins with the lipase-mediated release of α-linolenic acid (**2**) from the membrane lipids of chloroplasts (Fig. 1).[1] In chloroplasts, lipoxygenase (LOX) oxidizes α-linolenic acid (**2**) into 13(*S*)-hydroperoxy octadecatrienoic acid (13-HPOT, **3**). 13-HPOT (**3**) is metabolized by allene oxide synthase (AOS) into an unstable allene oxide (12,13-EOT, **4**), which is cyclized by allene oxide cyclase (AOC) into *cis*-(+)-12-oxo-phytodienoic acid (OPDA, **5**). The AOC reaction provides two side chain configurations in the naturally occurring jasmonate structure. Reduction of the 10,11-double bond in OPDA (**5**) by OPDA reductase 3 (OPR3) then yields 3-oxo-2-

(2-*cis*-pentenyl)cyclopentane-1-octanoic acid (OPC-8:0, **6**). Three β-oxidation steps convert OPC-8:0 (**6**) into (+)-7-*iso*-JA (**7**), which is naturally isomerized to (−)-JA (**1**). JA (**1**) is converted to the isoleucine conjugate of JA (JA-Ile, **8**) by JAR1. JA-Ile (**8**) is considered a versatile signaling compound in the JA signaling pathway.[1,3] JA-Ile (**8**) binds to its receptor, coronatine insensitive 1 (COI1), and then mediates the binding of the JAZ protein to the COI1-JA-Ile unit of the skp-cullin-F box (SCF) complex, resulting in degradation by the 26S proteasome and the subsequent induction of COI1-dependent JA responses.[4-6] OPDA (**5**) is not only an intermediate in the JA biosynthetic pathway but also exerts individual JA (**1**)-independent biological functions.[7-9] OPDA (**5**) binds cyclophilin 20-3, leading to enhanced redox capability in *Arabidopsis thaliana*.[10] In contrast, OPDA (**5**), but not JA (**1**), is present in the model bryophytes *Marchantia polymorpha* and *Physcomitrella patens,* with functions in defense and development.[11-13] However, the detailed mechanism of the OPDA signaling system remains unknown.

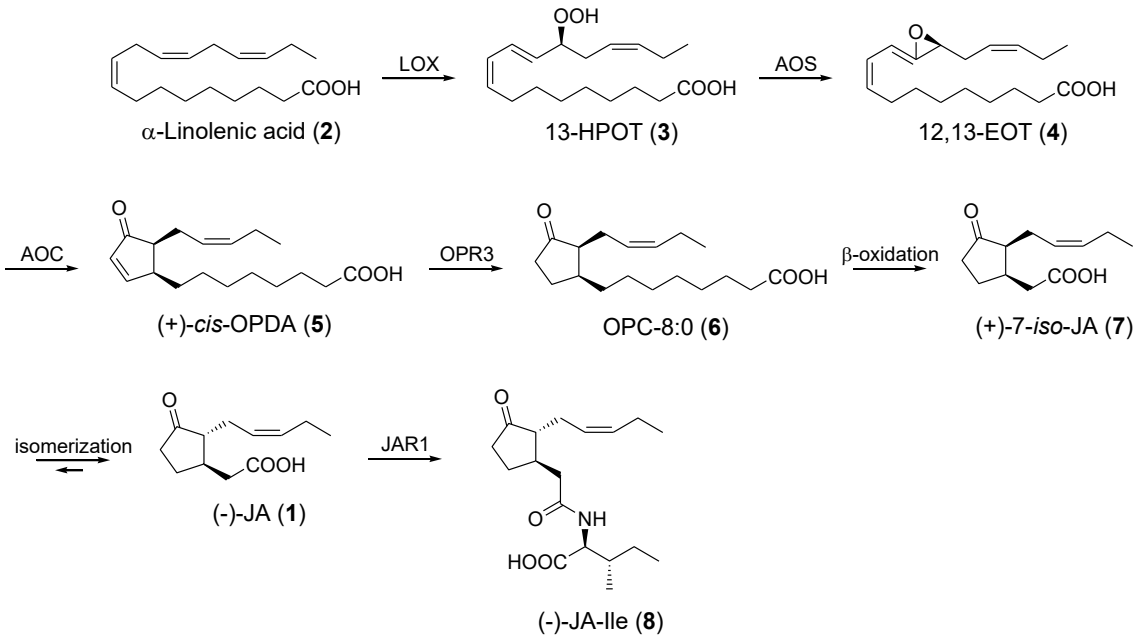
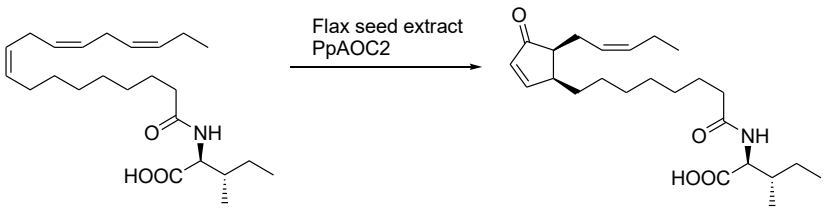


Fig. 1. Octadecanoid pathway.

OPDA-Ile (**9**), a new member of the jasmonate family, was recently identified in *A. thaliana*.[14] Moreover, OPDA-Ile (**9**) induces the expression of the *ZAT10* gene, which encodes a salt tolerance

zinc finger protein, and the *GRX480* gene, which encodes a GLUTAREDOXIN.[15,16] Based on these findings, OPDA-Ile (**9**) may function as a signaling molecule in plants. The OPDA-Ile (**9**) biosynthetic mechanism has not yet been determined, whereas the *A. thaliana jar1* mutant, which lacks the *jar1* gene encoding a protein that catalyzes the conjugation of JA (**1**) with Ile, produces OPDA-Ile (**9**).[16] Thus, the OPDA-Ile (**9**) biosynthetic pathway, which is independent of JAR1, is proposed to be present in *A. thaliana.*

The biological functions of OPDA-Ile (**9**) remain elusive. An efficient method for synthesizing OPDA-Ile (**9**) should be developed to investigate the detailed biological activities of this compound. OPDA-Ile (**9**) was previously produced via the chemical conjugation of Ile and OPDA (**5**) under alkaline conditions.[16,17] The stereochemistry of the two side chains of OPDA (**5**) is easily converted from the *cis*-form to *trans*-form under alkaline conditions; therefore, the previously reported method for synthesizing OPDA-Ile (**9**) is not necessarily optimal. For OPDA (**5**) biosynthesis, reactions with LOX, AOS and AOC occur on the unsaturated alkyl chains of α-linolenic acid (**2**), 13-HPOT (**3**), and 12,13-EOT (**4**), respectively.[18-20] Analysis of the crystal structures of AOS and AOC suggests that unsaturated alkyl chains of 13-HPOT (**3**) and 12,13-EOT (**4**) are present in the active sites of the corresponding enzymes.[18,19]



LA-Ile (**10**) OPDA-Ile (**9**)

Fig. 2. *In vitro* enzymatic synthesis of OPDA-Ile (**9**). LA-Ile (**10**) was incubated in the reaction mixture [50 mM Tris-HCl (pH 8.0), flax seed extract, PpAOC2] at 25 °C for 1 hours.

We attempted the *in vitro* cyclization of LA-Ile (**10**) to produce OPDA-Ile (**9**) by performing continuous reactions with LOX, AOS, and AOC according to the method for *in vitro* stereoselective OPDA (**5**) synthesis (Fig. 2).[21] The mixture used for the *in vitro* synthesis of OPDA-Ile (**10**) contained flaxseed extract, recombinant PpAOC2 derived from the model moss *Physcomitrella patens*, and LA-

Ile (**10**) and was incubated at 25 °C for 1 hour. As a result, 11 mg of OPDA-Ile (**9**) was successfully synthesized from 30 mg of LA-Ile (**10**) with a 35% yield (Supplemental data). Analysis of the AOS crystal structure suggests that a lysine residue of AOS near the substrate interacts with the carboxyl group of 13-HPOT (**3**), thereby playing an important role in its binding.[18] While the carboxyl group in linolenic acid (**2**) is replaced by an amide bond in LA-Ile (**10**), a lysine residue near the substrate of AOS may interact with the oxygen of the amide bond in a possible LOX product of LA-Ile (**10**). The alkyl chain of Ile moiety derived from LA-Ile (**10**) must not interfere with binding to LOX, AOS, or AOC. Therefore, the cyclization of LA-Ile (**10**) into OPDA-Ile (**9**) is found to have occurred. Additionally, the *in vitro* enzymatic synthesis of OPDA-Ile (**9**) was conducted under mild conditions and efficiently yielded OPDA-Ile (**9**). Considering the mechanisms of the LOX, AOS, and AOC reactions, the method reported in this study could be applied to the synthesis of other amino acid conjugates of OPDA.

The biosynthetic mechanism of OPDA-Ile (**9**) was not revealed until recently. Two possibilities exist for the OPDA-Ile (**9**) biosynthetic pathway. One possibility is that OPDA-Ile (**9**) is synthesized by a protein that conjugates OPDA (**5**) and Ile. In the case of JA-Ile (**8**), a GH3 protein, JAR1 conjugates JA (**1**) and Ile.[1] A protein from the GH3 protein family is predicted to catalyze the conjugation of OPDA (**5**) and Ile. The other possibility is that OPDA-Ile (**9**) is biosynthesized by three continuous reactions with LOX, AOS, and AOC with LA-Ile (**10**) as the substrate, similar to the *in vitro* enzymatic synthesis of OPDA-Ile (**9**). In a previous study, the marginal conversion of OPDA into OPDA-Ile was observed in WS.[15] The *in vitro* enzymatic synthesis of OPDA-Ile (**9**) in this study supports the hypothesis that OPDA-Ile (**9**) is biosynthesized from LA-Ile (**10**) via LOX-, AOS-, and AOC-mediated reactions in plants. *A. thaliana* plants grown for 30 days under short-day conditions were treated with 100 μM LA-Ile (**10**) or OPDA (**5**), and the accumulation of OPDA-Ile (**9**) was analyzed by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Based on the analytical data, the application of LA-Ile (**10**) significantly increased the OPDA-Ile (**9**) concentration in *A. thaliana*. The amount of OPDA-Ile (**9**) in plants treated with LA-Ile (**10**) was greater than that in untreated plants (Fig. 3). In contrast, the OPDA (**5**) treatment did not increase the OPDA-Ile (**9**) concentration (Fig. 3). These results suggested that OPDA-Ile (**9**) was biosynthesized

from LA-Ile (**10**) but not OPDA (**5**).

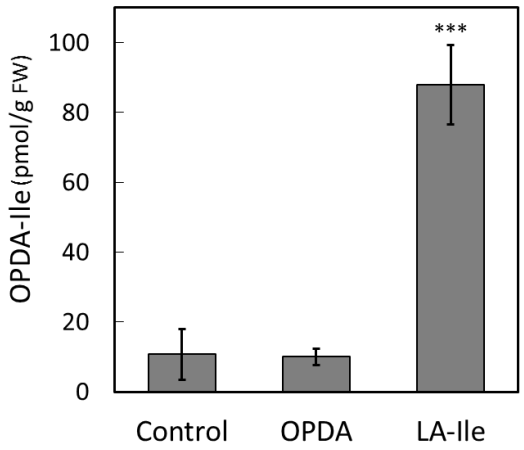


Fig. 3. UPLC-MS/MS analysis of OPDA-Ile (**9**) in *A. thaliana* treated with OPDA (**5**) or LA-Ile (**10**). Plants were treated with either 100 μM LA-Ile (**10**) or OPDA (**5**). OPDA-Ile (**9**) was analyzed by UPLC-MS/MS. The MRM mode was used to analyze a specific fragment peak at *m/z* 130.00 $[M-H]^-$ derived from the peak at *m/z* 404.28 $[M-H]^-$. Each value is represented by the mean ± SD of five independent biological replicates. Student's *t*-test, ***$p < 0.001$.

As described above, OPDA-Ile (**9**) was postulated to be converted from LA-Ile (**10**); however, LA-Ile (**10**) has not been reported as a natural product. *A. thaliana* was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine the presence of LA-Ile (**10**). The analytical data revealed a predominant peak derived from LA-Ile (**10**) in the chromatogram of an *A. thaliana* extract (Fig. 4). The retention time of the peak was the same as the peak for the LA-Ile (**10**) standard. Thus, LA-Ile (10 pmol/g FW, **10**) is present in *A. thaliana*. To our knowledge, this report represents the first evidence identifying LA-Ile (**10**) as a natural product.

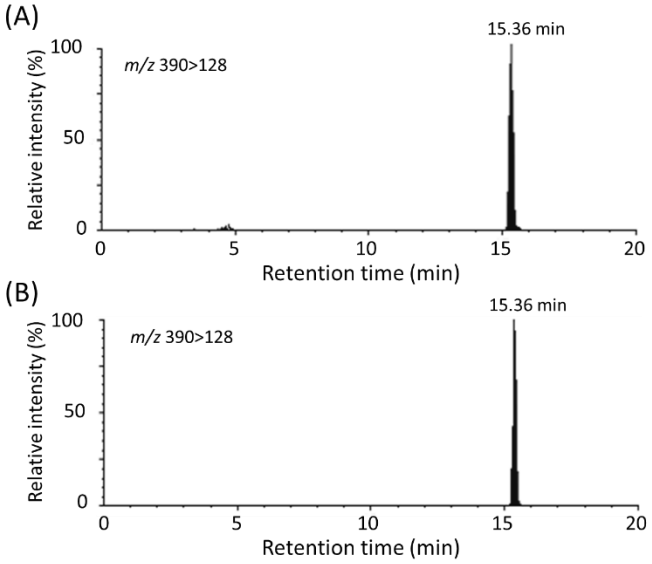


Fig. 4. Analysis of LA-Ile (**10**) in *A. thaliana.* LA-Ile (**10**) was analyzed by LC-MS/MS. The MRM mode was used to analyze a specific fragment peak at *m/z* 128.0 $[M-H]^-$ derived from the peak at *m/z* 390.3 $[M-H]^-$. (A): standard; (B): plant extract.

To examine whether OPDA-Ile (**9**) was synthesized by an *A. thaliana* protein extract, a reaction mixture comprising LA-Ile (**10**) and an *A. thaliana* protein extract was incubated for 1 hour, and then the reaction mixture was analyzed for the presence of OPDA-Ile (**9**) by UPLC-MS/MS. The peak derived from OPDA-Ile (**9**) appeared clearly in the reaction mixture of LA-Ile (**10**) and the protein extract (Fig. 5). In contrast, no clear peak derived from OPDA-Ile (**10**) was detected in the protein extract lacking LA-Ile (**10**) or in the buffer used to generate the protein extract supplemented with LA-Ile (**10**) (Fig. 5). *Arabidopsis* protein extract was shown to exhibit sequential LOX, AOS, and AOC enzymatic activities to convert LA-Ile (**10**) into OPDA-Ile (**9**).

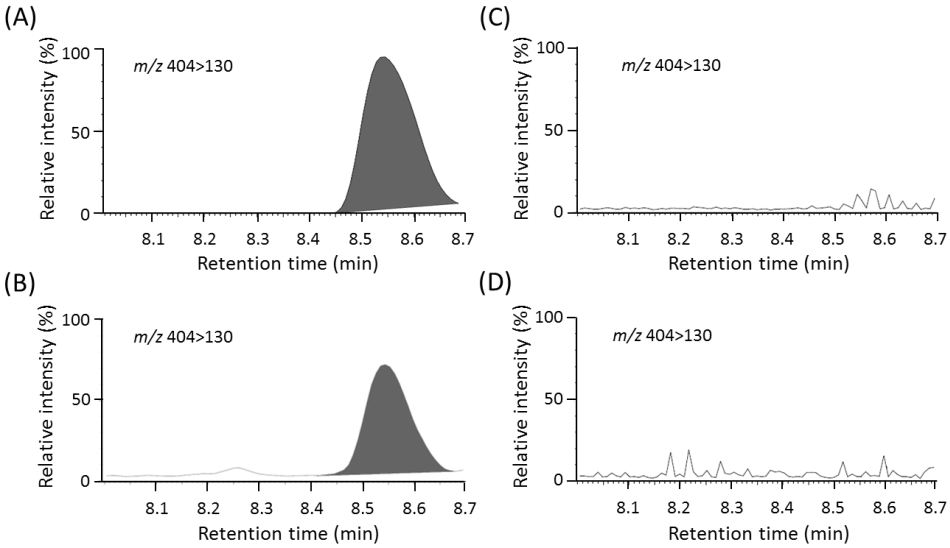


Fig. 5. *In vitro* synthesis of OPDA-Ile (**9**) by protein extracts from *A. thaliana.* LA-Ile (**10**) was incubated with a protein extract prepared from *A. thaliana* at 25 °C for 1 hour, and the mixture was then analyzed for OPDA-Ile (**9**) by UPLC-MS/MS. The MRM mode was used to analyze a specific fragment peak at *m/z* 130.00 $[M-H]^-$ derived from the peak at *m/z* 404.28 $[M-H]^-$. (A): standard OPDA-Ile (**9**); (B): LA-Ile (**10**) in protein extract; (C): protein extract without added LA-Ile (**10**); (D): LA-Ile (**10**) in the buffer used for protein extraction.

Next, we investigated whether stable-isotope-labelled LA-Ile (**9**) was incorporated into OPDA-Ile (**9**). LA-[$^{13}C_6$,^{15}N]Ile was fed to *A. thaliana*, and OPDA-[$^{13}C_6$,^{15}N]Ile in *A. thaliana* was then analyzed by UPLC-MS/MS. The analytical data showed that the peak derived from OPDA-[$^{13}C_6$,^{15}N]Ile (*m/z* 411>137) clearly appeared, and the retention time of OPDA-[$^{13}C_6$,^{15}N]Ile was in accordance with that of non-labelled OPDA-Ile (*m/z* 414>130) (Fig. 5). Accordingly, OPDA-Ile (**9**) is biosynthesized via cyclization of the LA-Ile (**10**) substrate in *A. thaliana.*

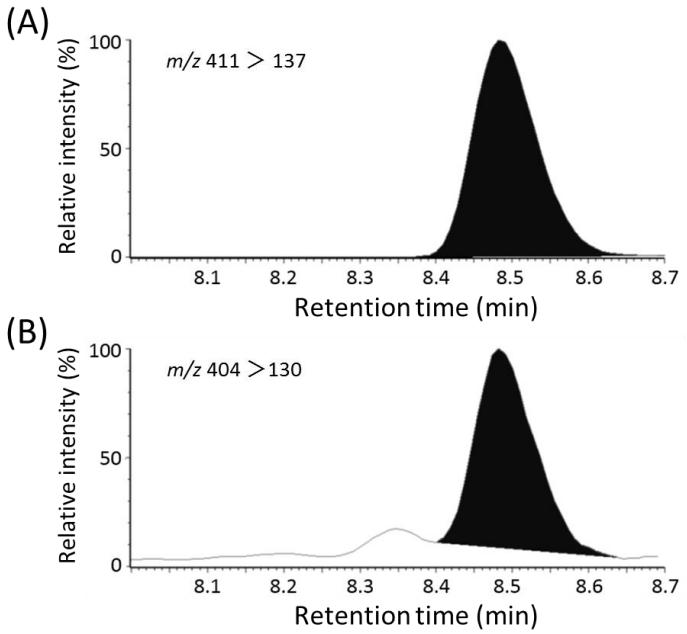


Fig. 5. Incorporation of LA-[$^{13}C_6$,^{15}N]Ile into OPDA-[$^{13}C_6$,^{15}N]Ile into *A. thaliana*. Plants were treated with or without 100 μM LA-[$^{13}C_6$,^{15}N]Ile, and the resulting mixture was analyzed for OPDA-[$^{13}C_6$,^{15}N]Ile by UPLC-MS/MS. The MRM mode was used to analyze a specific fragment peak of OPDA-[$^{13}C_6$,^{15}N]Ile at *m/z* 137.00 $[M-H]^-$ derived from the peak at *m/z* 411.28 $[M-H]^-$ and a specific fragment peak of OPDA-Ile at *m/z* 130.00 $[M-H]^-$ derived from the peak at *m/z* 404.28 $[M-H]^-$. (A); extract of plant treated with LA-[$^{13}C_6$,^{15}N]Ile: (B); extract of control plant.

Based on the data described above, OPDA-Ile (**9**) is biosynthesized in *A. thaliana* via LOX-, AOS-, and AOC-mediated reactions, which participate in the octadecanoid pathway, using LA-Ile (**10**) as the substrate. This result is supported by previous studies showing that the *jar1* mutant still produces OPDA-Ile (**9**), that the *aos* mutant does not produce OPDA-Ile and that marginal conversion of OPDA into OPDA-Ile occurs in *A. thaliana.*[14,15] Because the three proteins, LOX, AOS and AOC, are localized in chloroplasts, OPDA-Ile (**9**) is predicted to be located in chloroplasts. Additionally, arabidopsides, monogalactosyl glycerol lipids containing OPDA,[20] are likely synthesized by a combination of LOX-, AOS-, and AOC-mediated reactions using monogalactosyldiacylglycerol as the substrate.[22] The previously reported data also support the results obtained in this study. Thus, the

present study suggests that an α-linolenic acid-related compound with a modified carboxylic acid can become a substrate in the octadecanoid pathway. It is possible that a variety of OPDA-related compounds are biosynthesized through the octadecanoid pathway in plants.

As described above, LA-Ile (**10**) is a synthetic precursor of OPDA-Ile (**9**). LA-Ile (**10**) synthesis is also a crucial step in OPDA-Ile (**9**) biosynthesis; however, the conjugation mechanism of α-linolenic acid (**2**) and Ile in *A. thaliana* remains unclear. As GH3 proteins conjugate amino acids and plant hormones, such as indole-3-acetic acid and JA (**1**),[23] a member of the GH3 protein family likely plays an important role in OPDA-Ile (**10**) biosynthesis. The identification of an enzyme that catalyzes the conjugation of α-linolenic acid (**2**) and Ile is required to elucidate the total biosynthetic pathway for OPDA-Ile (**9**).

Acknowledgments

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Supplementary data

The supplementary data associated with this article can be found in the online version.

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Supplemental data

1. General methods

The ^{1}H- and ^{13}C-NMR spectra were recorded on a Jeol EX-270 NMR spectrometer (Jeol, Tokyo, Japan). ^{1}H-NMR chemical shifts are referenced to the residual $CDCl_3$ solvent peak at δ7.24 ppm. ^{13}C-NMR chemical shifts are referenced to the residual $CDCl_3$ solvent peak at δ77.0 ppm. Field desorption-high resolution mass spectra (FD-HR-MS) were recorded on a JEOL JMS T100GCV mass spectrometer (Jeol, Tokyo, Japan). Specific rotation values were measured on a JASCO DIP-310 polarimeter (Jasco Corporation, Tokyo, Japan).

2. LA-Ile (**10**) synthesis

α-Linolenic acid (**2**, 0.88 mmol, 245 mg) was dissolved in tetrahydrofuran (11 ml) with trimethylamine (0.98 mmol, 0.14 ml). Chloroformic acid ethyl ester (0.1 mmol, 0.1 ml) was added while the mixture was stirred at −10 °C. After the resulting solution was stirred for 20 min, a 0.3 M aqueous NaOH solution (6.9 ml) containing Ile (1.77 mmol, 232 mg) was added to the solution and stirred for an additional 25 min at room temperature. After evaporation to remove the solvent, the obtained residue was cooled at 0 °C, and poured into 1 M HCl, extracted with ethyl acetate, and dried over Mg_2SO_4. The extract was evaporated and purified by SiO_2 gel column chromatography (Kanto Chemical, Tokyo, Japan), which was developed with a mixed solvent of acetic acid/ethyl acetate/*n*-hexane (1/30/70, v/v). LA-Ile (**10**) was obtained as a colorless oil (293.2 mg, 85%). FD-HR-MS: found *m/z* 392.3150 $[M+H]^+$; calculated *m/z* 392.3165 for $C_{24}H_{42}NO_3$; $[\alpha]^{25}_{D}$ +19.8 (*c* 0.3, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 270 MHz) δ: 11.03 (s, 1H), 6.44 (d, *J* = 8.6 Hz, 1H), 5.38-5.21 (m, 6H), 4.59 (dd, *J* = 4.6, 8.6 Hz, 1H), 2.77-2.73 (m, 4H), 2.22 (t, *J* = 8.1 Hz, 2H), 2.08-1.85 (m, 6H), 1.60-1.38 (m, 3H), 1.22-1.07 (m, 8H), 0.97-0.81 (m, 9H).

3. LA-[$^{13}C_6$,^{15}N]Ile synthesis

Instead of Ile, [$^{13}C_6$,^{15}N]Ile was conjugated to a-linolenic acid. The reaction was carried out according to the method described in the previous section.

306

307 4. OPDA-[$^{13}C_6$,^{15}N]Ile synthesis

308 Instead of LA-Ile (**10**), LA-[$^{13}C_6$,^{15}N]Ile was cyclized to afford OPDA-[$^{13}C_6$,^{15}N]Ile according to
309 the method described in the previous section.

310

311 4. *In vitro* enzymatic synthesis of OPDA-Ile (**9**)

312 OPDA-Ile (**9**) was synthesized *in vitro* using LA-Ile (**10**) as the substrate, according to the method
313 used for the *in vitro* synthesis of OPDA (**5**) (Kajiwara et al., 2012). The reaction mixture used for the
314 *in vitro* synthesis of OPDA-Ile (**9**) contained a flaxseed extract, recombinant PpAOC2 and LA-Ile (**10**).
315 An acetone powder of the flaxseed extract (625 mg) was extracted with 5 ml of 50 mM Tris-HCl buffer
316 (pH 8.0, 20 mM NaCl) containing 500 μg of recombinant PpAOC2 and then centrifuged at 21,500 ×
317 *g* for 30 min at 4 °C. The prepared enzyme solution was incubated with 30 mg of LA-Ile (**10**) and
318 stirred under an oxygen atmosphere for 3 hours at room temperature. The reaction mixture was
319 extracted with ethyl acetate. After the extract was evaporated, the resulting residue was purified by
320 SiO_2 gel column chromatography, which was developed with a mixed solvent of acetic acid/ethyl
321 acetate/*n*-hexane (1/30/70, v/v) to obtain 11 mg of OPDA-Ile (**9**). $[\alpha]^{25}_D$ +42.3 (*c* 0.6, $CHCl_3$). FD-
322 HR-MS: found *m/z* 404.2804 [M-H]; calculated *m/z* 404.2801 for $C_{24}H_{38}NO_4$. ^{1}H-NMR ($CDCl_3$, 270
323 MHz) δ: 7.74-7.70 (dd, *J* = 5.8, 2.6 Hz, 1H), 6.20-6.12 (dd, *J* = 3.1, 1.8 Hz, 1H), 6.08-5.98 (d, *J* = 8.3
324 Hz, 1H), 5.48-5.28 (m, 2H), 4.64-4.54 (dd, *J* = 5.6, 4.8 Hz, 1H), 3.03-2.88 (m, 1H), 2.55-2.46 (m, 1H),
325 2.46-2.36 (m, 1H), 2.26-2.17 (t, *J* = 7.4 Hz, 2H), 2.15-2.07 (m, 1H), 2.06-1.96 (m, 2H), 1.96-1.84 (m,
326 1H), 1.77-1.65 (m, 1H), 1.64-1.54 (m, 2H), 1.53-1.40 (m, 1H), 1.34-1.24 (m, 6H),1.23-1.09 (m, 2H),
327 1.05-0.72 (m, 11H). ^{13}C-NMR ($CDCl_3$, 67.5 MHz) δ: 208.8, 172.4, 171.1, 165.0, 130.5, 129.8, 124.3,
328 53.8, 47.4, 41.8, 35.1, 34.0, 28.1, 27.0, 26.6, 26.5, 25.0, 23.1, 22.6, 21.2, 18.2, 12.7, 11.4, 9.0.

329

330 5. Plants and chemical treatments

331 *A. thaliana* (Col-0) was grown on soil under short day conditions (10 hours of light/14 hours of
332 dark) at 25 °C for 30 days under a white fluorescent light. Plants were sprayed with 100 μM OPDA
333 and LA-Ile once per day for 3 days. For the feeding experiment with LA-[$^{13}C_6$,^{15}N]Ile, *A. thaliana* was

harvested at 12 hours after spraying 100 μM LA-[$^{13}C_6$,^{15}N]Ile. OPDA was synthesized according to the method reported by Kajiwara et al. (2012).

6. Analysis of OPDA-Ile (**9**)

A. thaliana plants were grown on Jiffy-7 (Sakata Seed Corporation, Yokohama, Japan) for 5 weeks at 22 °C under a white fluorescent light with 10 h/14 h photoperiods (short-day conditions). Samples were prepared according to the method developed by Floková et al. (Phytochemistry. 2016; 122: 230–237). UPLC was performed using an ACQUITY UPLC system (Waters Corporation, Milford, MA, USA) equipped with a binary solvent manager and a sample manager. MS/MS was subsequently performed using a Micromass Quattro Premier tandem quadrupole MS (Waters Corporation, Milford, MA, USA). The UPLC/MS system was controlled by Micromass MassLynx 4.0 (Waters Corporation, Milford, MA, USA). The UPLC conditions were described previously (Sato et al., Plant Cell Physiol. 2011; 52: 509-517). The MS parameters for the detection of OPDA-Ile were set according to the method described by Floková et al. (Phytochemistry. 2016; 122: 230–237). OPDA-[$^{13}C_6$,^{15}N]Ile was used as a standard for quantitative analysis.

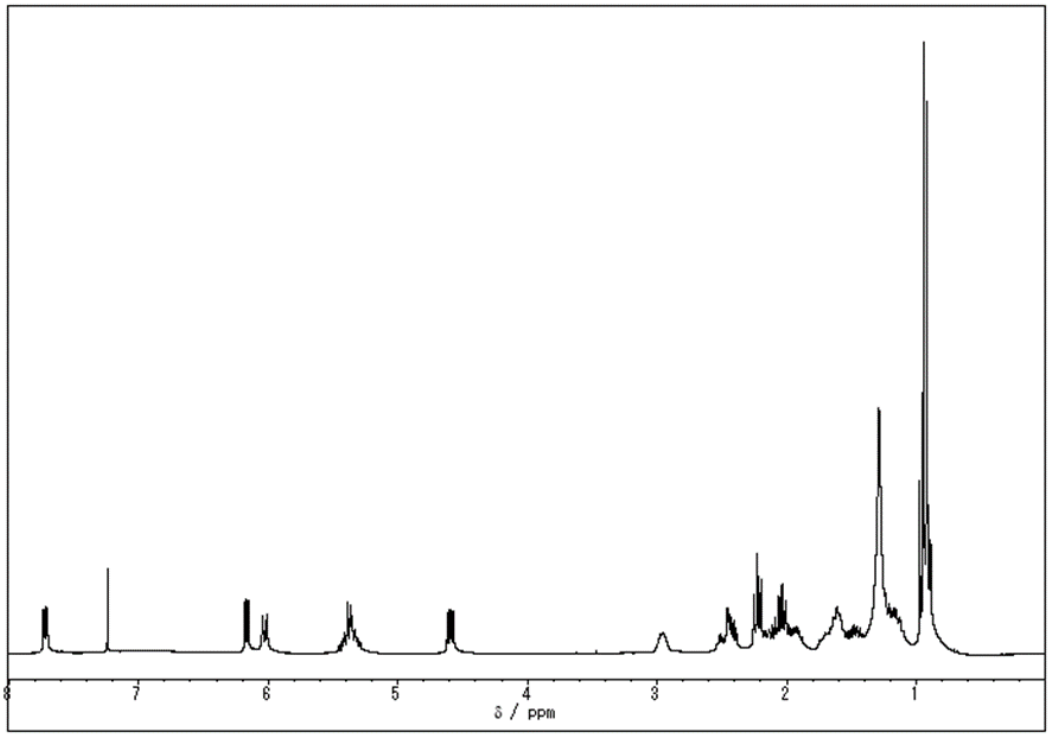
7. Analysis of LA-Ile (**10**)

A. thaliana plants were grown according to the method described in the previous section. Plants (500 mg) were then extracted with 10 ml of an 80% aqueous MeOH solution. The resulting extract was applied onto a C18 solid phase extraction cartridge (Bond Elut, 6 ml, Agilent Technologies, CA, USA) that had been equilibrated with 80% aqueous MeOH. After the cartridge was washed with 6 ml of 80% MeOH, LA-Ile (**10**) was eluted with 6 ml of MeOH. The eluate was evaporated, and the obtained residue was dissolved in 500 μl of 80% aqueous MeOH for analysis. The detection and quantification of LA-Ile (**10**) were performed using a 4000Q TRAP LC-MS/MS system (Sciex, Framingham, MA, USA) equipped with an electrospray ionization (ESI) source (turbo V) and 1290 Infinity HPLC system (Agilent, Santa Clara, CA, USA). Chromatographic separation was performed at 40 °C on a TSK-gel ODS-100V column (150 mm × 2 mm inner diameter (i.d.), 5 μm) (Tosoh Corporation, Tokyo, Japan). Eluents were composed of water/formic acid (99.9/0.1, v/v)

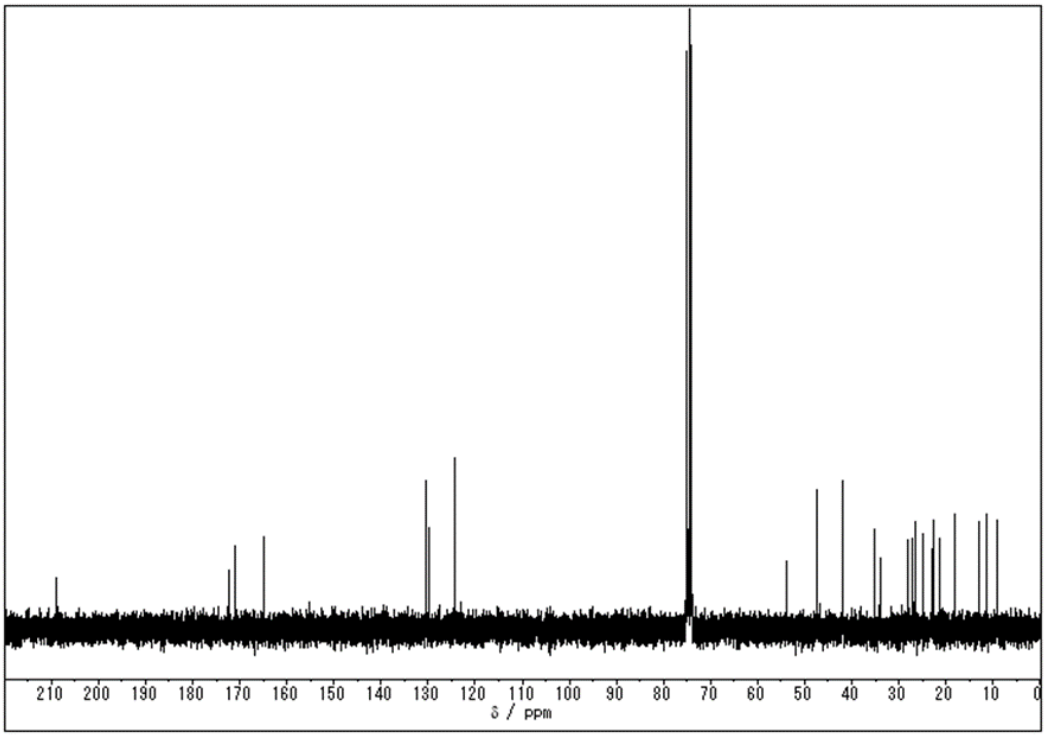
(eluent A) and methanol/formic acid (99.9/0.1, v/v) (eluent B). Elution was conducted at a flow rate of 0.20 ml/min with the following linear gradient: 0–3 min, 50% B; 3–18 min, 50–97% B; 18–22 min, 97% B; 22–22.1 min, 97–50% B; and 22.1–29 min, 50% B. The injection volume was 10 µl. MS data were acquired in multiple reaction monitoring (MRM) mode. The conditions of the interface were as follows: ion spray voltage, −4500 V; source temperature, 450 °C; curtain gas pressure, 10 psi; nebulizing gas pressure, 70 psi; and turbo gas pressure, 80 psi. The parameters used for the mass spectrometry of LA-Ile (**10**) are listed in Supplemental Table S1. Analyst 1.6.2 software was used for data acquisition and processing. LA-[$^{13}C_6$,^{15}N]Ile was used as a standard for quantitative analysis. The values given are the mean ± SD of five independent biological replicates.

8. *In vitro* synthesis of OPDA-Ile (**9**) by an *A. thaliana* protein extract

Plants (1.0 g) were ground in liquid nitrogen and extracted with 10 ml of 100 mM sodium phosphate buffer (pH 7.8). The residue was removed by centrifugation at 20,000 × *g* for 15 min, and the supernatant was then used as a protein extract to synthesize OPDA-Ile (**9**). One milliliter of the protein extract supplemented with 1 mM LA-Ile (**10**) was incubated at 25 °C for 1 hour. The pH of the reaction solution was adjusted to approximately 3, and the solution was next extracted with an equal volume of ethyl acetate and then evaporated. The resulting residue was dissolved in 200 µl of 80% aqueous MeOH, and OPDA-Ile (**9**) was analyzed by UPLC-MS/MS according to the method described above. A protein extract without added LA-Ile (**10**) and 100 mM sodium phosphate buffer (pH 7.8) supplemented with LA-Ile (**10**) were used as controls.



Supplemental Fig. S1. ^{1}H-NMR spectrum of OPDA-Ile (**9**) (270 MHz, $CDCl_3$).



Supplemental Fig. S2. ^{13}C-NMR spectrum of OPDA-Ile (**9**) (67.5 MHz, $CDCl_3$).

Supplemental Table S1. Optimized MS parameters for the analysis of LA-Ile.

Compound	Scan mode	MRM transition	Declustering potential (V)	Entrance potential (V)	Collision energy (V)	Collision exit potential (V)
LA-Ile	–	390.3/128.0	-80	-10	-34	-19